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A Simplified Procedure for Determination of Metallothionein in Animal Tissues[1)]

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(Received December 24, 1977)

Since metallothionein binds strongly with cadmium at a definite molar ratio, an examination was made on the method for quantitative determination of metallothionein using this characteristic.

1) Free cadmium was removed by heating with a guinea pig hemolysate and subsequent separation by centrifugation.

2) Zinc in the metallothionein fraction was completely substituted with cadmium by its addition and treatment of this fraction with the guinea pig hemolysate resulted in approximately complete removal of cadmium other than that in the metallothionein fraction.

3) This method showed a good linearity in the wide range of cadmium content of 0.2 to 4.0 μg/ml.

4) Result obtained from this method was compared with the existing method of measuring the amount of metal (s) bound to the metallothionein fraction after gel filtration in the liver and kidneys of rats given cadmium, and the values were in approximate agreement. Correlation coefficient was 0.98 for the liver and 0.95 for the kidneys.

Keywords——environmental pollution ; determination of metallothionein ; cadmium ; substitution of metal ; hemolysate treatment ; animal tissue.

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1) Presented October 1976 at the 26th Kinki Chapter Meeting of the Japanese Pharmaceutical Society held in Sayama.

2) Location: Igawadani-cho, Tarumi-ku, Kobe.

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Foreword

As contamination of the environment by heavy metals such as Cd, Hg is increasingly becoming a serious problem over the last years, there has been concern regarding the behaviour of these metals inside the body, and it was demonstrated that the greatest part combined inside the living body with micromolecular proteins rich in SH groups, metallothionein[3, 4] (abbreviated hereafter as MT). As the administration of these heavy metals induces the biosynthesis of MT[5] and as the Cd bound to MT does not decrease the enzymatic activity[6], it seems that this protein inhibits the toxic action caused by the heavy metals. It was also reported that Cd administered in the form of MT causes more kidney disorder than when administered in the form of free Cd[7]. A detailed examination is therefore required.

From such vantage point, one may expect that a proven simple quantitative analysis method for MT should play an important role in explaining the toxicity caused by heavy metals. However, the traditional quantitative analysis method was merely performed on the basis of the amount of heavy metals in the MT fractions obtained through gel-filtration. Apart from this, the sole method tested is that of Piotrowski et al.[8] who used ^{203}Hg.

We reported that heavy metals bind with MT at a certain molar ratio but that there were differences in bonding characteristics with MT depending on the various types of metal, and that MT combined more strongly with Cd than with Zn, Hg[9]. So we thought that it would be easy to determine the amount of MT from the amount of bound CD if all the MT bound metals were replaced with Cd. We report here on our examination of such measurement method.

Method of the tests

1. Preparation of the hemolysate

The hemolysate was prepared as follows. We added 10 mL of 2% double oxalic acid* solution to 21 mL of guinea pig blood, centrifuged for 10 min at 3,000 rpm and washed the obtained precipitation with 30 mL of 2% double oxalic acid* solution. Thereafter, we added 20 mL of distilled water and heamolyzed the erythrocytes. We centrifuged this solution for 5 min at 3,000 rpm and added 0.03 M Tris-HCl buffer solution (pH: 8.0) (abbreviated hereafter as Tris B) to the obtained supernatant. This was used as hemolysate.

2. Removal of free Cd by hemolysate (hemolysate treatment)

To remove the free Cd in the internal organ supernatant containing MT, we added hemolysate diluted with Tris B, cooled with water after heating for 3 min at 80°C and obtained a supernatant through centrifugation at 3,000 rpm for 5 min. We performed this operation as hemolysate treatment. We normally repeated it 3 times.

3. Preparation of (domestic) rabbit liver supernatant containing MT

We added a 5-volume 0.25 M sucrose solution to the liver of male (domestic) rabbit injected twice hypodermically with a 3 mg Cd/kg dose of $CdCl_2$ solution (3 mg Cd/mL) and homogenized. We centrifuged this homogenate at 105000 x g for 60 min and obtained rabbit liver supernatant (abbreviated hereafter as RLS). The MT fractions obtained by gel-filtration of 1 mL of RLS contained 18.1 μg of Cd and 10.1 μg of Zn.

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*Note - this is a literal translation. Since oxalic acid normally exists as $(COOH_2).2H_2O$ we assume the dihydrate is referred to.

4. Cd and Zn measurements

We measured Cd and Zn by the direct atomization method using a 208-model Hitachi atomic absorption photometer.

Results

1. Free Cd removal through hemolysate treatment

When computing the amount of MT from the amount of Cd bound to the MT fraction with the addition of Cd to internal organ supernatant containing MT, it is necessary to remove the Cd not bound to the MT fraction. So we first examined the effectiveness of the hemolysate treatment on the free Cd. Since the hemolysate seems to contain virtually no MT we first added Tris B to 1 mL of Cd solution (10 μg/mL) so as to have a final volume of 4 mL and measured the Cd of the supernatant, treating once with hemolysate diluted to 1-1/500 (Fig. 1).

Although the Cd volume decreased in proportion to the volume of added hemolysate when diluting the hemolysate to 1/50 or lower the removal was insufficient. When diluting to up to 1/50, the Cd volume was extremely low and we saw that it was possible to separate the added 10 μg of free Cd. This is why we subsequently used in the experimental hemolysate treatment 0.2 mL of 20-times-diluted hemolysate per one treatment.

2. Effect of hemolysate treatment with regard to RLS

We examined whether or not it was possible to remove the Cd not bound to the MT fraction through hemolysate treatment. We added 0.1 mL of Cd solution (1000 μg/mL) to 1 mL of RLS as well as Tris B so as to have a final volume of 4 mL, and reacted for 5 min at room temperature. Treating

this reaction solution with hemolysate, we obtained a supernatant, gel-filtered with Sephadex G-50, and measured the Cd and Zn. For the gel-filtration, we used a 1.9 x 42 cm column and eluted with Tris B. The eluate was divided into fractions of 4 mL each. We compared the results obtained when not using this elution pattern with that obtained without the hemolysate treatment (Fig. 2).

When no hemolysate treatment is performed, the added Cd was eluted not only in the MT fraction as reported before[9] but also into the macromolecular and micromolecular fractions. When performing the hemolysate treatment, we noted a little Cd on the MT fraction and in the micromolecular fraction. The Cd not bound to the MT fraction could be removed almost entirely. We saw no Zn in this MT fraction and the amount of bound Cd agreed practically with the calculated values obtained assuming that all the Zn in the MT fraction was replaced by Cd.

3. <u>The range of quantitative determination</u>

The above results suggest that the hemolysate treatment removes the Cd outside the MT fraction without affecting the MT combined with Cd and thus to calculate the MT amount by measuring the Cd amount by such method. We therefore examined the range within which MT can be quantitatively determined.

We added 1 mL of Cd solution (10 µg/mL) to various volumes of RLS as well as Tris B so as to have a final volume of 4 mL. We performed the hemolysate treatment and measured the Cd concentration of the supernatant (Fig. 3).

We noted that there is a linear relationship between the RLS volume and the Cd concentration in the supernatant after hemolysate

treatment. A good linearity was exhibited throughout the wide Cd 0.2-4.0 μg/mL range.

4. MT addition-recovery test

In order to ascertain whether the present method could be applied to the various internal organs, we performed addition-recovery tests on 10 types of internal organs. We excised the liver, kidneys, spleen, heart, stomach, lungs, testes, brain, small intestine, and muscle from 450 g normal Wistar male rats. We added 5-volumes of 0.25 M sucrose solution to the various internal organs and homogenized. We centrifuged the homogenate for 10 min at 10,000 rpm and obtained a supernatant. To 0.2 mL of this supernatant we added 0.05 mL of RLS, 1 mL of Cd solution (10 μg/mL) and 2.15 mL of Tris B. After hemolysate treatment, we measured the Cd concentration in the supernatant. We substracted from these values the values obtained without RLS addition and compared with the Cd of MT in RLS (Table 1).

As a result, the values were a little low in the testes and brain at 88%, but were about 95% in all other eight types of internal organ.

5. Quantitative determination of MT in the liver and kidneys of rats treated with Cd

We injected hypodermically four groups of two 200-250 g male Wistar rats each with 3, 6, 9, 12 mg of Cd per kg of body weight as $CdCl_2$ solution. We sacrificed the rats 48 h later, added 5-volumes of 0.25 M cane sugar solution to the liver and kidneys, and homogenized. We centrifuged the homogenate for 10 min at 10,000 rpm and used the supernatant as sample.

To 0.2 mL of liver supernatant we added 1 mL of Cd solution (10 μg/mL), 2.2 mL of Tris B and reacted for 5 min at room temperature. We measured the Cd concentration of the supernatant after hemolysate treatment. Since 1 mL of liver supernatant was bound to 70-120 nmol Cd we compared with the values obtained by the traditional method, that is gel-filtering the liver supernatant and calculating the sum of the molar numbers of Cd and Zn which are the metals bonded with the MT fractions (Fig. 4). There is practical agreement between the values of both methods. When we examined the correlation, we found a positive correlation. The correlation coefficient was 0.98.

With the kidneys, we used 0.5 mL of supernatant for a similar examination (Fig. 5). Here also we obtained a positive correlation. The correlation coefficient was 0.95.

Discussion

Piotrowski et al[8] reported on an internal standard method for MT determination using ^{203}Hg. There seems however to be some weaknesses: the half life of ^{203}Hg is short viz. 46.9 days, highly purified MT is required for assay and the bonding strength of Hg toward thionein is weaker than that of Cd. Moreover, possibly because of the admixture of Hg not originating from MT inside the "MT fraction" used, this method yields abnormally high MT concentration values for the liver and kidneys of the healthy animals measured.

So, we tried to make quantitative measurements of MT from the amount of bound Cd. Since Hg, Zn bonded to MT can easily be replaced by Cd, we first substituted Cd for the metals in MT by adding excess Cd. Thereafter, we removed by hemolysate treatment the Cd not bonded with MT.

As a result, the Cd determination range in the present method was as large as 0.2-4.0 μg/mL. Even when comparing with the traditional method calculating from the amount of metal bound with the MT fraction after gel-filtration, a practical agreement was demonstrated. Moreover, as the reproducibility and yield rate are good, the present method is fast and simple in assaying the MT derived in the liver, kidneys and such of animals treated with heavy metals.

We noted some differences in the MT metal composition and contents which depend on the material used for purification. There are reports of 11% (w/w) Cd and 0.1% Zn for Form I, and of 5% Cd and 4% Zn for Form II of MT in domestic rabbits[10]. In all cases, when substituting with Cd, both forms contain about 11% Cd in weight ratio. Consequently, if the present method is used, one may multiply by 9.0 the amount of bonded Cd when calculating the amount of MT. Moreover, since Cu binds more strongly with thionein than Cd, the present method cannot be used in the presence of Cu ions or for MT containing a large amount of Cu.

When making quantitative determinations of MT in the internal organs of healthy rats using the present method, we obtained relatively high values for the kidneys, testes, brain and small intestine (Table 1). So after adding ^{109}Cd to 4 types of internal organ supernatant we performed the hemolysate treatment after which we performed gel-filtration. In all cases ^{109}Cd eluted into the macromolecular fraction but we noted also a peak in the MT fraction. This fact suggests that even though the quantitative determination of trace MT in the internal organs of healthy animals is difficult, detection is possible. We intend to establish later a method using ^{109}Cd to determine microquantities of MT.

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*Translator's note: reading not quite certain.

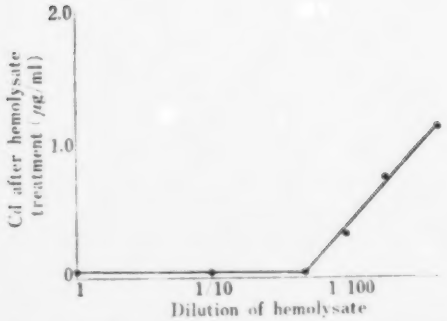

Fig. 1. Effect of Hemolysate Concentration on the Removal of Free Cadmium

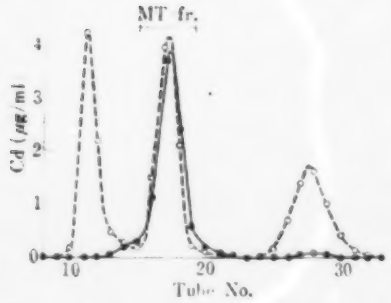

Fig. 2. Gel Filtration of Cd Added Rabbit Liver Supernatant on Sephadex G-50 Column

Hemolysate-treated, —●—: Cd.
None-treated, ---○---: Cd.

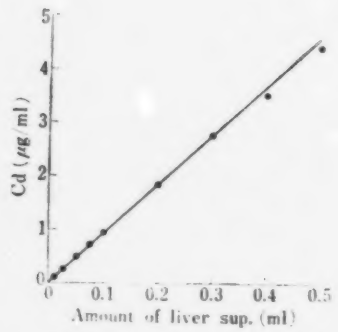

Fig. 3. Determination of MT in Rabbit Liver Supernatant by the Hemolysate Treatment Method

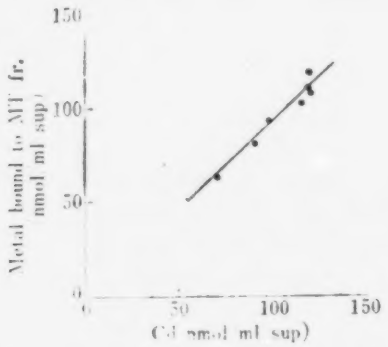

Fig. 4. Relationship between Sephadex G-75 Method and Hemolysate Treatment Method for the Determination of MT in Rat Liver Supernatant

$\gamma = 0.98$.

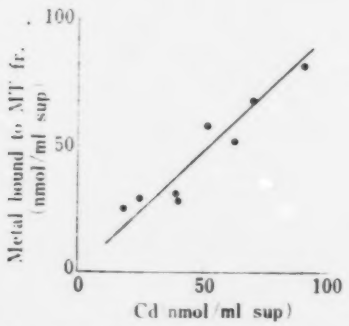

Fig. 5. Relationship between Sephadex G-75 Method and Hemolysate Treatment Method for the Determination of MT in Rat Kidney Supernatant

$\gamma = 0.95$.

TABLE I. Recovery of MT in Various Tissues of Rat by Hemolysate Treatment Method

Tissue	Tissue (Cd μg/ml)	Tissue + RLS (Cd μg/ml)	MT (Cd μg/ml)	MT recovered (%)
RLS			0.51	100
Liver	0.01	0.49	0.48	94
Kidney	0.09	0.56	0.47	92
Spleen	0.02	0.50	0.48	94
Heart	0.00	0.49	0.49	96
Stomach	0.01	0.50	0.49	96
Lung	0.01	0.51	0.50	98
Testis	0.17	0.62	0.45	88
Brain	0.10	0.55	0.45	88
Small intestine	0.06	0.55	0.49	96
Muscle	0.00	0.49	0.49	96